

REVIEW ARTICLE

***Fusobacterium nucleatum* Infection in Colorectal Cancer: Linking Inflammation, DNA Mismatch Repair and Genetic and Epigenetic Alterations**

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Abstract:

It has been recently reported that the population of *Fusobacterium*, particularly *Fusobacterium nucleatum* (*Fn*), is overrepresented in colorectal cancers (CRCs) and adenomas. The promoting effects of *Fn* infection on adenoma and/or carcinoma formation have been shown in *Apc*^{Min/+} mice. Characteristics of *Fn*-associated CRC were identified through studies using human CRC cohorts and include right-sided colon location, CpG island methylation phenotype (CIMP)-high, high level of microsatellite instability (MSI-H), and poor patient prognosis. A subset of *Fn*-associated CRC exhibits a low level of microsatellite instability (MSI-L) and elevated microsatellite alterations in selected tetra-nucleotide repeats (EMAST) induced by translocation of MSH3 from the nucleus to the cytoplasm in response to oxidative DNA damage or inflammatory signals. The association between CIMP/MSI-H and *Fn* infection can be explained by the role of the mismatch repair protein complex formed between MSH2 and MSH6 (MutS α) to repair aberrant bases generated by reactive oxygen species (ROS) to form 7,8-dihydro-8-oxo-guanine (8-oxoG). Clustered 8-oxoGs formed at CpG-rich regions including promoters by ROS is refractory to base excision repair. Under these conditions, MutS α initiates repair in cooperation with DNA methyltransferases (DNMTs) and the polycomb repressive complex 4. DNMTs at damaged sites methylate CpG islands to repress transcription of target genes and promote repair reactions. Thus, continuous generation of ROS through chronic *Fn* infection may initiate (1) CIMP-positive adenoma and carcinoma in an MSH2/MSH6-dependent manner and/or (2) MSI-L/EMAST CRC in an MSH3-dependent manner. The poor prognosis of *Fn*-associated CRC can be explained by *Fn*-induced immune-evasion and/or chemoresistance.

Keywords:

microsatellite instability-high (MSI-H), elevated microsatellite alterations at selected tetra-nucleotide repeats (EMAST), colorectal cancer (CRC), mismatch repair (MMR), *Fusobacterium nucleatum*, CpG island methylator phenotype (CIMP)

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Introduction

Microbiota imbalances in the colon and rectum are associated with an inflammatory microenvironment and promotion of colorectal cancer (CRC)¹. Several bacterial organisms including *Fusobacterium nucleatum* (*Fn*), Enterotoxigenic *Bacteroides fragilis* (*ETBF*), and colibactin-producing

Escherichia coli (*E. coli*) are epidemiologically associated with CRC and have been found to be enriched in CRC^{1,2}. Dejea *et al* showed that CRCs in the ascending colon and hepatic flexures but not on the left side of the colon are covered with invasive bacterial films³. The enrichment of *Fn* was found in colorectal adenomas relative to non-adenomas or surrounding normal tissues^{4,6}. Recent studies further dem-

onstrated that the CRCs with a high load of *Fn* infection is associated with a high level of microsatellite instability (MSI-H) and a high level of CpG island methylation phenotype (CIMP-H) CRC and is associated with a right-side location and poor prognosis^{7,8}. There is also a subgroup of non-MSI-H CRC that exhibit microsatellite alterations in response to inflammatory tumor-microenvironment^{9,10}. For this review, we have compiled articles that deal specifically with *Fn* infection and CRC. On the basis of data in these articles, we discuss possible mechanisms for genetic and epigenetic change induced by *Fn* and the possible role of *Fn* in the prognosis of CRCs.

Discovery of *Fusobacterium* in Colorectal Neoplasms

Fn is one of 14 species belonging to the genus *Fusobacterium*. It is Gram-negative and anaerobic and is associated with various infections including periodontitis, otitis media, Lemierre's disease, amniotic fluid infections and inflammatory bowel disease^{11,12}. *Fn* is a common resident in the human gut mucosa and is one bacteria that colonizes CRC tumors more frequently than adjacent normal mucosa^{11,13}. Clear evidence for the abundance of *Fn* in CRC tumor tissues compared with nearby normal tissues was first reported by two studies in 2012^{14,15}. Kostic *et al* used a whole genome sequence approach where they identified microbial DNA sequences by subtracting human DNA sequences from total DNA reads. They found an enrichment of genus *Fusobacterium* in most CRC tissues (8/9 cases). They further amplified microbial 16S ribosomal-DNA and sequenced from 95 tumor/normal CRC pairs and also performed fluorescence *in situ* hybridization to show that *Fusobacterium* is enriched in tumor tissues. They identified *Fn* as the most enriched species among *Fusobacterium* species in CRC¹⁴. Castellarin *et al* used an RNA-seq approach where human transcript sequences were subtracted from total RNA reads in 11 paired CRC tumor/normal tissues. They found enrichment of *Fn* in tumors (more than twofold) as compared with matching normal tissues (9/11 cases). They developed a quantitative PCR assay to determine *Fn*-specific copy numbers using the *nusG* gene of *Fn* and measured *Fn* copies in genomic DNA from 99 CRC tumor/normal tissues. Again, *Fn* was enriched in tumors compared with normal tissues. They also showed that *Fn* isolated from one CRC patient was invasive to cultured colon cancer cells. Finally, they showed that a high level of *Fn* in CRC was associated with lymph node metastasis¹⁵. Later, an enrichment of *Fn* in CRC tumor tissues was found in cohorts from Japan⁶, Europe¹⁶, and China¹⁷, indicating that this phenomenon is universal among human populations.

McCoy *et al* first described the association between *Fusobacterium* and colorectal adenoma⁴. They compared the

abundance of *Fusobacterium* in normal rectal mucosa isolated from subjects with adenoma (48 cases) and from subjects without adenoma (67 cases) and found that rectal mucosa from adenoma patients were significantly enriched in *Fusobacterium* compared with mucosa from subjects without adenoma⁴. In contrast to McCoy's study, Kostic *et al* directly quantified *Fusobacterium* spp. loads in adenomas and matched adjacent normal tissues from the same patient. They found significant enrichment of the *Fusobacterium* species in adenoma tissues ($P < 0.004$)⁵. Flanagan *et al* quantified *Fn* loads in 52 colorectal adenomas and nearby normal mucosa and found that there was a trend but no significant association between the *Fn* level and adenoma state. However, they found a significantly higher *Fn* load in high-grade dysplasia and in CRC, thus suggesting that the *Fn* level was associated with progression of adenoma to carcinoma¹⁶. Although a higher level of *Fn* load was detected in adenoma and carcinoma tissues compared with adjacent normal tissues, carcinoma tissues contained more *Fn* than adenoma tissues¹⁶. Similarly, Yu *et al* observed a gradual increase in the *Fn* load during the transition from adenoma to carcinoma¹⁸. Taken together, these results suggest that *Fn* infection may be involved in progression from adenoma to carcinoma in some CRC cases.

Mouse Model of *Fn*-associated Intestinal Tumorigenesis

To evaluate *Fn* infection in intestinal tumorigenesis, Kostic *et al* used C57BL/6 *Apc*^{Min/+} mice, which develop intestinal tumors because of an inactive mutation in one copy of the *Apc* gene⁵. Oral introduction of *Fn* (13 of 15 mice) but not of control *Streptococcus* spp. (two of 12 mice) or tryptic soy broth (one of 20 mice) significantly increased the number of colonic tumors in *Apc*^{Min/+} mice. Kostic *et al* also replicated observations found in human tissues that of enrichment of *Fn* in tumor tissues compared with surrounding normal mucosa and invasiveness of *Fn* to tumor tissues.

Yu *et al* used both C57BL/6 and C57BL/6 *Apc*^{Min/+} mice models¹⁸. In the C57BL/6 model, mice were treated with a chemical carcinogen, 1,2-dimethylhydrazine (DMH), followed by introduction of *Fn* or control bacteria (*E. coli*). Introduction of *Fn* increased the number of DMH-induced aberrant crypt foci and tumors compared with introduction by *E. coli*. Similar to Kostic's results, introduction of *Fn* increased the number of tumors in C57BL/6 *Apc*^{Min/+} mice¹⁸. Similarly, Yang *et al* showed that introduction of *Fn* into C57BL/6 *Apc*^{Min/+} mice increased the number and size of tumors and shortened overall survival compared with the non-treated controls¹⁹. Although these three studies suggest that *Fn* infection may promote loss of a wild-type copy of *Apc* in *Apc*^{Min/+} colon cells to form adenoma, they do not give evidence that *Fn* infection may be involved in adenoma-to-

carcinoma transition in the colon and rectum. It would be interesting to see the genetic and epigenetic landscapes of the *Fn*-induced tumors.

In contrast to the three studies discussed above, Tomkovich *et al* did not see any tumor-enhancing effects of *Fn* on *Apc*^{Min/+} mice²⁰. They used germ-free (GF) or GF-derived, specific-pathogen-free (SPF) 129/SvEv *Apc*^{Min/+} mice, whereas other studies used SPF C57BL/6 *Apc*^{Min/+}. Thus, the mouse genetic background and/or different microbial exposures before and/or after *Fn* infection may change the susceptibility of *Apc*^{Min/+} mice to *Fn*. There was also a difference in the strain of *Fn* used by Tomkovich's study as compared with others. These discrepancies highlight the possibility that (1) there may be individual genetic and/or epigenetic backgrounds that are susceptible for *Fn*-associated tumorigenesis and/or (2) there may be a select group of *Fn* that are a component of diverse microbiota associated with colorectal tumorigenesis^{20,21}.

Characteristics of *Fusobacterium*-associated CRCs

Following the discovery of the association between *Fusobacterium* infection and CRC, it was recognized that the infection is significantly associated with two subgroups of CRCs. The first group of CRCs exhibit a high level of CpG island methylation phenotype (CIMP-H) and/or a high level of microsatellite instability (MSI-H) and are located on the right side of the colon⁶⁻⁸. The second group of CRCs exhibit a low level of microsatellite instability (MSI-L) and/or elevated microsatellite alterations in selected tetra-nucleotide repeats (EMAST) and are also located on the right side of the colon (unpublished data).

CIMP and MSI-H CRCs

Tahara *et al* found that 74% of tumor tissues from 149 CRC cases were tested positive for infection by the *Fusobacterium* species, including *Fn* (52.3%;78/149). Among infected CRCs, 14 (9.4%) and 8 (5.4%) cases were heavily infected with *Fusobacterium* spp. or *Fn*, respectively. They further showed that the *Fusobacterium*-high CRCs were enriched in MSI-H ($P = 0.018$), CIMP-positive ($P = 0.001$), *hMLH1* methylation-positive ($P = 0.0028$), p53 wild-type ($P = 0.015$), and CDH7/8 mutant CRCs ($P = 0.002$) and located on the right side⁷. Ito *et al* also showed that 56% of tumor tissues from 511 cases of CRC were positive for *Fn* infection. They divided *Fn*-infected groups into high and low on the basis of the median number of *Fn* bacteria present and found that *Fn*-high was associated with tumor tissues rather than premalignant tissues ($P = 0.0001$), larger tumor size ($P = 0.0005$), and CIMP-high ($P = 0.0013$)⁶. Mima *et al* also showed that a high load of *Fn* in CRC tissues was associated with MSI-H, CIMP, and *BRAF* mutation by uni-

variate analysis but not by multivariate analysis adjusted for MSI-H²².

The human cancer genome exhibits aberrant DNA methylation^{23,24}. Increased methylation of promoter region CpG islands is prominent in tumor DNA and is associated with transcriptional inactivation of tumor-suppressor genes. Toyota *et al* first systematically examined CpG islands that were differentially methylated between tumor and normal genomic DNA from CRC patients²⁵. They found a group of the CpG sites specifically associated with the cancer state in a subset of CRC and gave this phenotype the name *CpG island methylation phenotype* (CIMP). Thus, CRC can be divided into CIMP-positive (CIMP⁺) and CIMP-negative (CIMP⁻). They further demonstrated that (1) CIMP is seen in a subgroup of adenomas; (2) CRC with MSI-H due to promoter hypermethylation of *hMLH1*²⁶ is a subgroup of CIMP⁺ CRCs; (3) *CDKN2A* and *THBS1* are frequently methylated in CIMP⁺ CRCs; and (4) CIMP⁺ CRC is associated with proximal site²⁵. Later, it was recognized that CIMP is not a dichotomous trait but rather continuous and can be divided into CIMP⁺, CIMP-intermediate, and CIMP⁻. Sánchez-Vega *et al* analyzed methylation data from The Cancer Genome Atlas (TCGA) CRC cohort (274 cases: reference 27) using CIMP⁺, CIMP-intermediate, and CIMP⁻ categories and confirmed that a large portion of CIMP⁺ exhibit hypermethylation of *hMLH1* (MSI-H) and are associated with the right side of the colon. They also showed that hypermethylation of *MGMT* is frequent in CIMP⁺ CRC and is associated with *FBXW7*, *APC*, and *KRAS* mutations²⁸.

Microsatellites, or simple sequence repeats, are composed of 1-6 repeated nucleotides. Microsatellite instability (MSI) is defined as continuous length changes in simple DNA repeats within microsatellite loci. MSI-H is caused by deficiencies in mismatch repair (MMR) genes including *MSH2*, *MLH1*, *MSH6*, and *PMS2*²⁹. As mentioned above, the MSI-H exhibited in 10-15% of sporadic CRC cases is due to transcriptional down-regulation of *MLH1* expression through promoter hypermethylation²⁶.

CRCs have been divided into several subgroups on the basis of their genetic and epigenetic landscapes²⁷, their transcriptional³⁰ or proteomic similarities or differences³¹, and their immunological landscapes³². In 2012, genetic and epigenetic landscapes of sporadic CRC identified through massive DNA/RNA sequencing were reported by the TCGA Consortium²⁷. One of the major findings was that there are two types of CRC that differ in respect to the frequency of somatic gene mutations. Sixteen percent of CRC showed a hypermutated genotype where three-quarters exhibited MSI-H because of *MLH1* silencing by promoter-methylation, and one-quarter had somatic mutations in the MMR gene and polymerase ϵ (POLE)²⁷.

The transcriptional landscapes in CRC can be divided into four distinctive subgroups: (1) the consensus molecular sub-

type 1 (CMS1) (14%) that is enriched in hypermutated and MSI-H CRC and characterized by gene expression with strong immune activation; (2) CMS2 (37%), marked with WNT and MYC signaling activation; (3) CMS3 (13%) with metabolic dysregulation; and (4) CMS4 (23%), marked with prominent transforming growth factor- β activation, stromal invasion, and angiogenesis. CRCs with mixed features (13%) also exist and may represent a transitional phenotype³⁰. Although most of MSI-H CRC is grouped into CMS1, some of them are grouped into CMS3 and CMS4³⁰. In proteomic subgrouping, there are five distinctive groups within the TCGA CRC cohort. Proteomic subtype B and C are enriched in MSI-H/CIMP⁺ and hypermutated CRC. These observations indicate that MSI-H/CIMP⁺ CRCs are heterogeneous in terms of m-RNA and protein expression^{30,31}. Angelova *et al* analyzed TCGA CRC data to identify a dominant immune cell subpopulation associated with a particular molecular subtype of CRCs. They found that tumor-infiltrating lymphocytes (TILs) from MSI-H CRC tumors are enriched by lymphocytes with antitumor functions including central and effector memory CD4⁺/CD8⁺ cells and natural killer T cells, compared with TILs from MSS CRC³².

MSI-L/EMAST CRCs

MSI in CRS was defined in 1998 at an international workshop meeting sponsored by the National Cancer Institute²⁹. In this meeting, MSI-H, low-frequency of MSI (MSI-L), and microsatellite stable (MSS) CRCs were defined using a specific panel of microsatellite markers. Although MSI-H is caused by the functional loss of MMR proteins, the etiology of MSI-L and the distinction between MSI-L and MSS CRC remained unclear until recently^{10,29}. Another type of microsatellite alteration, termed EMAST, where insertion/deletion mutations in the loci with tetra-nucleotide but not with mononucleotide and/or dinucleotide repeats, was recognized as a component of CRC^{33,34}. MSI-L and EMAST have been observed in many human cancers^{10,33}. Haugen *et al* examined the frequency of EMAST in CRC, its relationship to MSI-L, and its possible causes³⁴. They found that EMAST is frequent in sporadic cases of non-MSI-H CRC (~50%) and is associated with decreased nuclear MSH3 expression in tumor cells. Using isogenic MSH3-proficient and MSH3-deficient cell lines, they also showed that EMAST and MSI-L in non-MSI-H CRC cells are caused by loss of MSH3³⁴. The frequent incidence of EMAST in CRCs was confirmed by two other studies^{35,36}. The fact that EMAST is due to the loss of MSH3 was also proven by other studies using tissue cultured human cells^{37,38}. Later, Adam *et al* found patients with bi-allelic inactivation mutations at the MSH3 locus who suffered from colorectal adenoma polyposis syndrome. The adenoma polyps from these patients exhibited MSI at loci with dinucleo-

tide repeats and EMAST loci but not at loci with mononucleotide repeats³⁹. These results strongly suggest that MSI-L/EMAST is caused by loss of the MSH3 function. The biochemical basis for MSI-L/EMAST formation through loss of MSH3 has been described elsewhere¹⁰.

Immunohistochemical staining using anti-MSH3 showed that nuclear MSH3 is completely lost in adenoma polyps with bi-allelic germline MSH3 mutations³⁹, whereas localized loss of nuclear MSH3 is detected in sporadic CRCs exhibiting MSI-L/EMAST^{34,36}. These results suggest that loss of MSH3 expression in sporadic MSI-L/EMAST CRC may be due to a somatic epigenetic event. The frequency of MSH3 somatic mutations in CRC is about 6.6%²⁷, which does not explain the high incidence of MSI-L/EMAST (~50%) in CRC.

Lee *et al* first showed evidence that inflammation may be linked to MSI-L/EMAST in CRC³⁶. They found that EMAST CRC is enriched in CD8⁺ T cells in the tumor microenvironment compared with non-EMAST CRC. They also found that EMAST is significantly high in ulcerated tumors. These results suggest that some immunological and inflammatory responses are active in EMAST CRC. Later, Tseng-Rogenski *et al* demonstrated that in several cancer cell lines^{38,40}, inflammatory factors including oxidative stress (hydrogen peroxide), interleukin-6 (IL6), and prostaglandin E₂ (PGE₂) induce displacement of MSH3 from the nucleus to the cytoplasm, whereas the other MMR proteins do not displace. Repeated treatment of microsatellite stable colon cancer cell lines with IL6 induced EMAST. These studies convincingly showed that some inflammatory factors induce EMAST through loss of MSH3 in the nucleus.

Evidence that the inflammatory microenvironment induces MSI-L had been found in regenerated colon tissues from ulcerated colitis (UC) patients. Brentnall *et al* showed for the first time the presence of MSI-L but not MSI-H in colon tissues from UC patients⁴¹. Ozaki *et al* also examined crypts from UC-derived CRC, UC-derived hyperplasia, and UC-regenerated colons and tested them for the presence of microsatellite instability. They again detected MSI-L but not MSI-H in some crypts but not in stroma cells regardless of whether they were from cancer or non-cancer tissues⁴². Recently, we have shown that regenerated colon epithelial cells and tumors from UC patients show a high frequency of MSH3 displacement from the nucleus to the cytoplasm and of MSI-L/EMAST. These results support the role of inflammation in displacement of MSH3, which induces MSI-L/EMAST in human tissues including cancers⁴³.

Our recent investigations not only confirmed that *Fn* is associated with MSI-H but also showed that *Fn* is associated with MSI-L/EMAST when compared with non-MSI-H, non-MSI-L/EMAST CRC (*unpublished data*). These findings raise the possibility that inflammatory factors generated in tumor-microenvironments in response to *Fn* infection and/or

Fn itself may induce translocation of MSH3 from the nucleus to the cytoplasm in colon tumor cells, resulting in MSI-L/EMAST.

Poor Prognosis for patients with *Fn*-associated CRCs

Increased loads of *Fn* are associated with advanced CRC stages^{6,16-18} and with poor prognoses^{19,22,44-46}. Mima *et al* showed that colon cancer-specific death, but not overall survival, is associated with high levels of tissue *Fn*²². Yu *et al* looked at the relationship between CRC recurrence and microbiota alterations in the gut. They found that *Fn* is the most enriched bacteria species in tumors from recurrent CRC compared with tumors from non-recurrent CRC. Using additional cohorts, they showed that the higher load of *Fn* is an independent determinant and predictor of recurrence-free survival (RFS)⁴⁴. Yan *et al* also showed that a high level of *Fn* in stages III and IV CRC is associated with shorter cancer-specific survival and shorter RFS⁴⁵. They also observed that adjuvant chemotherapy is more effective in preventing recurrence in stage III patients with a low level of *Fn* compared with those with a high level of *Fn*⁴⁵. Bullman *et al* showed that patients from TCGA cohorts who suffered from right-side colon cancer with a high load of *Fn* infection had poor overall survival compared with patients with a low load of *Fn* infection. Interestingly, they detected *Fn* infection not only in primary CRC but also in matching liver metastasis. They also demonstrated that antibiotic treatment not only reduced the *Fusobacterium* load but also inhibited growth of xenograft tumors from patients, suggesting that infection by *Fusobacterium* accelerates tumor growth *in vivo*⁴⁶.

Possible Explanation of Why *Fusobacterium* Infection is Associated with CIMP CRC

CIMP has been detected in many human cancers, but its causes are not clear²⁸. Several observations suggest that infection by microorganisms and/or inflammation associated with microorganism infection may induce CIMP in tumor genomes. For instance, in gastric cancers, there are two types of CIMP⁺ tumors: one is associated with Epstein-Barr virus (EBV) infection, and another is associated with MSI-H (*hMLH1* promoter hypermethylation)⁴⁷. Infection of *Helicobacter pylori* in gastric cells also induces inflammation that causes aberrant DNA methylation⁴⁸. In fact, when human cell lines were co-cultured with *Fn* *in vitro*, expression of pro-inflammatory genes including *TNF*, *IL8*, and *IL1 β* as well as ROS was induced^{49,50}. Kostic *et al* analyzed transcriptome sequences from 133 cases of CRC in the TCGA data set²⁷ to identify the genes associated with *Fusobacterium* species infections⁵. They found that the expression of inflammatory response genes including *IL1 β* , *IL24*, *PTGS2* (*COX-2*), *IL8*, *IL6*, and *TNF* were enriched in

Fusobacterium-infected CRCs. Importantly, these gene signatures were specific to *Fusobacterium* but not to other bacteria detected in CRC tissues including *Bacteroides*, *Escherichia*, *Streptococcus*, and *Propionobacterium*⁵. They further demonstrated that *Fn*-induced tumors in *Apc*^{Min/+} mice express elevated levels of mouse homologs of *PTGS2*, *IL8*, *IL6*, and *TNF* genes compared with tumors not induced by *Fn*. Finally, they showed that NF- κ B, which drives the pro-inflammatory response, was activated in *Fn*-enriched CRC tissues⁵. Similarly, Yang *et al* reported that *Fn*-infection activates Toll-like receptor 4 (TLR4)/NF- κ B axis in cultured cells, and NF- κ B is highly activated in *Fn*-enriched CRC tissues¹⁹. These observations strongly suggest that the tumor-microenvironment of *Fn*-infected CRC is highly inflammatory.

One of the factors that would be constantly generated by chronic *Fusobacterium* infection and could be responsible for an aberrant DNA methylation is ROS⁵¹. Although there are many cases where ROS is associated with DNA hypermethylation^{48,52}, until recently, there has been no direct evidence that oxidative DNA damage causes a genome-wide hypermethylation of promoter CpG islands and hypomethylation of CG sites at other parts of the genome. The aberrant base, 7,8-dihydro-8-oxo-guanine (8-oxoG), is the most abundant DNA modification generated by ROS. Therefore, 8-oxoG would be enriched at promoter CpG islands within the whole genome after ROS exposure. Base excision repair (BER) is primarily responsible for removing 8-oxoG; however, clustered 8-oxoG lesions are known to be refractory to BER⁵³. Zlatanou *et al* first reported that MutS α , the heterodimer formed between the MMR proteins MSH2 and MSH6, recognizes and initiates removal of the clustered 8-oxoG in cooperation with mono-ubiquitinated PCNA and DNA polymerase ϵ ⁵³. Independently, O'Hagan *et al* discovered that a large protein complex consisting of DNA methyltransferase I (DNMT1), DNMT3B, and a member of the polycomb repressive complex 4 (PRC4), linked to transcriptional silencing, tightly binds to a promoter CpG island of the expressed gene when the island is damaged by ROS. Binding of this complex leads to a reduction of gene expression and induces DNA methylation at the CpG islands of the target gene⁵⁴. Ding *et al* successively demonstrated when MutS α recognizes and repairs 8-oxoG, it also recruits DNMT1 to the damaged site⁵⁵. Taken together, these results support the idea that enriched 8-oxoG at the promoter CpG islands attracts MutS α , and at the same time, DNMTs and PRC4 are recruited to reduce the transcriptional activity of the target gene through promoter methylation during MutS α -directed repair. This imbalance in DNMTs/PRC4 localization between promoter and non-promoter CpG sites explains how genome-wide hypermethylation of promoter CpG islands, CIMP, and hypomethylation of CG sites in other parts of the genome develop in response to ROS dam-

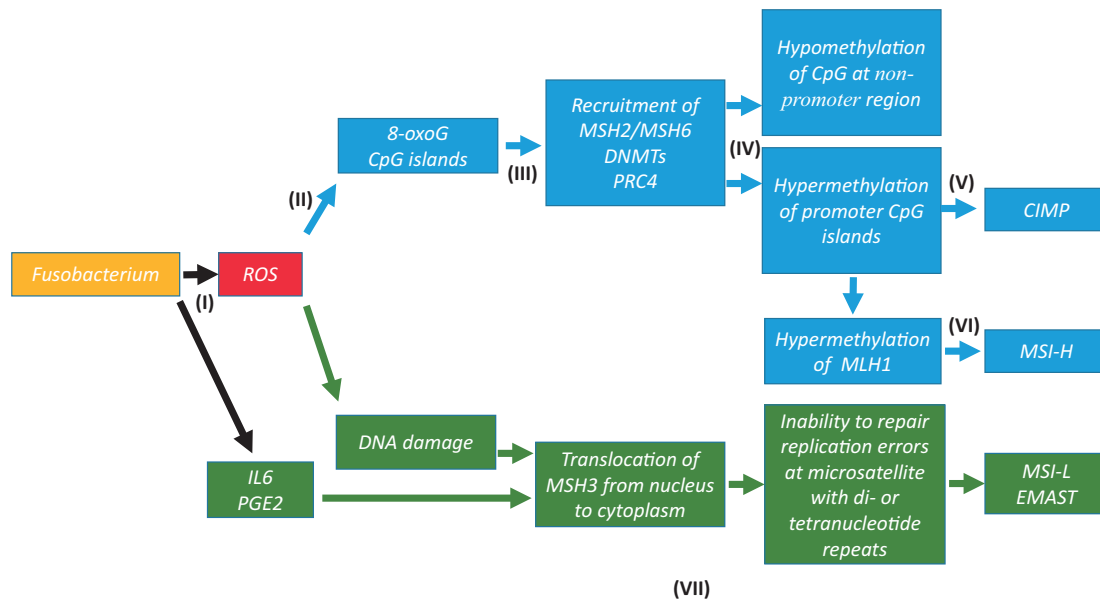


Figure 1. Hypothetical Pathways of Genetic and Epigenetic Alterations in CRC Induced by Chronic *Fusobacterium nucleatum* Infection.

(I): Chronic infection of colon mucosa by *Fn* induces ROS and other pro-inflammatory factors including IL6 and PGE₂ (references 5,50).

(II): ROS generates clustered 8-oxoG lesions at promoter CpG island.

(III): MSH2/MSH6, DNMT1, DNMT3B, and PRC4 are recruited from whole genome and enriched at damaged promoter CpG islands.

(IV): Non-promoter CpG sites become DNMT-poor, leading to hypomethylation.

(V): Recruited DNMT1 and DNMT3B methylates promoter CpGs to enhance DNA repair by MSH2/MSH6, leading to hypermethylation of CpG islands (CIMP) (references 53-56).

(VI): Hypermethylation of the *hMLH1* promoter CpG island leads to MSI-H.

(VII): DNA damage (8-oxoG) or IL6/PGE₂ induces translocation of MSH3 from nucleus to cytoplasm, leading to MSI-L/EMAST (references 38, 40).

Blue arrows and boxes represent the pathway to CIMP/MSI-H triggered by *Fn* infection.

Green arrows and boxes represent the pathway to MSI-L/EMAST triggered by *Fn* infection.

age in the cancer genome. Finally, this mechanism has been proven by Maiuri *et al* using a mouse model of colitis-associated CRC⁵⁶. They showed that inflammation-induced tumors gained DNA hypermethylation at CpG islands in an MSH2-dependent manner⁵⁶. They used *Min*^{Apc Δ 716^{+/+}} and *MSH2*^{fl}/VC/*Min* mice and inoculated mice with *Bacteroides fragilis* (ETBF) to generate colitis-associated CRC⁵⁷. They found that ETBF-induced tumors had more hypermethylated regions and fewer hypomethylated regions in their genome compared with ETBF-mock tumors. They observed a transient increase in the amount of 8-oxoG in colon epithelium cells from ETBF-infected mice and a synchronous reduction of gene expression from the loci that were hypermethylated in ETBF-induced tumors. They detected MSH2 interacting with DNMTs and components of PRC4 in ETBF-inflamed colon cells. Lastly, they demonstrated that loss of MSH2 reduces CpG island hypermethylation in ETBF-induced tumors⁵⁶.

Although the ETBF-induced mouse CRC model high-

lights a casual role of ROS in the CIMP phenotype, whether *Fn*-infection also activates MSH2/MSH6-dependent hypermethylation of promoter CpG islands in CRC remains to be determined (Figure 1).

Possible Explanation of Why *Fusobacterium* Infection is Associated with MSI-H CRC

Although the CpG island of the *MLH1* promoter would be one of many sites damaged by ROS and methylated by DNMTs/PRC4, resulting in MSI-H (Figure 1), it is not clear why inactivation of this locus has an advantage for CRC initiation triggered by inflammation. One possible explanation is that silencing *MLH1* may aid the damaged cells in escaping apoptosis induced by oxidative stress. Hardman *et al* showed that *MLH1*-deficient cells are more resistant to the cytotoxic effects of hydrogen peroxide or tert-butyl hydroperoxide than are *MLH1*-proficient cells. They observed a lack of apoptotic events including increased mitochondrial

permeability, release of cytochrome c, and caspase 3 activation in *MLH1*-deficient cells after exposure to hydrogen peroxide⁵⁸). Yanamadala *et al* confirmed Harman's results and showed that apoptosis induced by hydrogen peroxide is *MLH1*-dependent and associated with global inhibition of m-RNA⁵⁹). Chen *et al* found that *MLH1* is cleaved by caspase-3 during apoptosis activated by DNA damage and that this cleaved *MLH1* product plays a role in the execution of apoptosis⁶⁰). Thus, for colonic epithelial cells continuously exposed to ROS by chronic infection of *Fn*, silencing *MLH1* would be one way to avoid apoptosis.

Fusobacterium Infection is Associated with MSI-L/EMAST

As mentioned above, cumulative evidence supports the idea that the etiology of MSI-L/EMAST formation in cancer genome is the inflammation-induced loss of nuclear MSH3 in dividing cells^{10,38,40}). Therefore, it is reasonable to speculate that a subgroup of MSI-L/EMAST CRC may be associated with pathogenic infections of microbiota including *Fusobacterium*. We have examined a CRC cohort for the MSI-H and MSI-L/EMAST genotypes and for the level of *Fn* in each CRC case. We have confirmed that *Fn*-positivity is associated with MSI-H compared with non-MSI-H. We have also found that *Fn*-positivity is significantly associated with MSI-L/EMAST as opposed to non-MSI-H and non-MSI-L/EMAST CRC (*unpublished data*). We are currently determining whether direct infection by *Fn* induces MSI-L/EMAST in tissue cultured cells and/or in mouse models (Figure 1).

Furthermore, it is possible that MSI-L/EMAST CRC also exhibits CIMP. In support of this assumption, a subset of CIMP CRC exhibits hypermethylation of *MGMT*, which is associated with MSI-L CRC^{28,61}).

Possible Explanation of Why Fusobacterium Infection is Associated with Poor Prognosis of CRC

Enhancement of Tumor Cell Growth and Survival

Yang *et al* demonstrated that *Fn* directly promotes CRC cell growth and survival *in vitro* and *in vivo*. *Fn* activates NF- κ B through TLR4 signaling, resulting in up-regulation of microRNA-21 that could be a marker for poor clinical outcomes for CRC patients¹⁹). *Fn* secretes adhesion and FadA through which attachment and invasion of *Fn* to a host cell occurs⁶²). Rubinstein *et al* showed that FadA binds to E-cadherin on the host cell and activates β -catenin, resulting in oncogenic and inflammatory responses⁶³). They observed that (1) *Fn* and purified FadA stimulate cell growth in several CRC cell lines in an E-cadherin-dependent man-

ner; (2) FadA binds to E-cadherin on CRC cells and promotes *Fn* attachment and invasion via E-cadherin; (3) the binding of FadA to E-cadherin initiates β -catenin nuclear translocation and increases transcription of the downstream oncogenic pathway including T cell factors and Myc and cyclin D genes. Further internalization of the FadA/E-cadherin complex by endocytosis also activates inflammatory NF- κ B signaling. Thus, FadA on the surface of *Fn* stimulates tumor growth through bacterial attachment but also triggers the inflammatory response through bacterial invasion. There are many other uncharacterized *Fn* proteins that may control host-pathogen interactions⁶⁴). Further studies are required to identify and characterize these proteins in relation to CRC carcinogenesis.

Immune evasion

CRC infected with a high load of *Fn* is associated with MSI-H and poor prognoses. However, it is widely accepted that the prognosis of MSI-H CRC is better than for non-MSI-H CRC. Papat *et al* showed that MSI-H CRC exhibits better overall survival than does non-MSI-H CRC through meta-analysis of 32 studies examining 7642 cases including 1277 MSI-H tumors⁶⁵). As mentioned above, the tumor-microenvironment of MSI-H CRC is enriched in lymphocytes with antitumor functions including CD4+/CD8+ T cells and natural killer (NK) cells. A high immunoscore based on the abundance of CD3⁺ and CD8⁺ T cells at the central and invasion fronts of tumor tissues is associated with improved prognosis, and the majority of MSI-H cases have this high immunoscore³²). The reason for the improved prognosis of MSI-H CRC compared with non-MSI-H CRC is the presence of an antitumor T cell population in the tumor-microenvironment, elicited by continuous generations of frameshift neoantigens in hypermutated MSI-H CRC⁶⁶). However, Mlecnik *et al* showed that MSI-H CRC is heterogeneous and consists of at least two subgroups; the major group exhibiting a positive prognosis with high T cell activity and the minor group exhibiting poor prognosis with reduced T cell activity^{67,68}). Therefore, it could be that CRC with a high *Fn* load is a subgroup of MSI-H CRC with reduced T cell activity. Compatible with this assumption, Mima *et al* showed that CRCs with high levels of *Fn* exhibited a reduced density of CD3⁺ T cells in their tumor-microenvironments⁶⁹).

Reduced T cell activity in the tumor-microenvironment can be achieved by cancer cells through loss of HLA expression and/or disabling the antigen-processing machinery (APM). In fact, alterations in the HLA complex containing B2M and APM occur more frequently in MSI-H CRCs⁷⁰⁻⁷³). Thus, this immune evasion mechanism may partially explain the immunological heterogeneity seen in MSI-H CRC.

MSI-H tumors that have lost HLA or APM function are still targets of NK cells⁷⁴). *Fn* secretes several proteins in-

involved in binding to other microorganisms or to host cells and in invasion. Fap2 was first identified as an apoptosis-inducing protein for human lymphocytes^{75,76}. Gur *et al* showed that infection of cancer cells with *Fn* protects cancer cells from cytotoxic attacks of NK and/or T cells via Fap2-TIGIT (T cell immunoglobulin and ITIM domain) interactions⁷⁶. The NK cell activity is controlled by inhibitory and activating NK receptors. TIGIT is a one of the inhibitory NK receptors, expressed in all NK cells. When its ligands (for instance, Fap2) bind to TIGIT, NK cells stop killing their target cancer cells. Copenhagen-Glazer *et al* demonstrated that Fap2 co-aggregates other microorganisms and attaches to the mammalian cell surface by binding to galactose- and N-acetyl-d-galactosamine (Gal/GalNAc)⁷⁷. Abed *et al* showed evidence that enrichment of *Fn* in CRC tumor tissues, as opposed to normal tissues surrounding these tissues, may be due to selective binding of Fap2 to Gal/GalNAc, which is overexpressed on the surface of tumor cells⁷⁸. Thus, cancer cells coated by Fap2 would be protected from an NK attack.

Chemoresistance

Yu *et al* convincingly demonstrated that a higher load of *Fn* in CRC is associated with cancer recurrence after surgery. They reasoned that chemoresistance is a major factor for recurrence and proved that *Fn* induces cancer cells' chemoresistance through activation of autophagy⁴⁴. We previously showed that stage II/III MSI-L/EMAST CRC exhibit a shorter RFS compared with MSI-H or non-MSI-H, non-MSI-L/EMAST CRC^{79,80}. Because MSI-L/EMAST CRCs are infected by *Fn*, it is tempting to speculate that a majority of *Fn*-associated CRCs that exhibit poor prognoses, especially shorter RFS, could be MSI-L/EMAST CRCs but not MSI-H CRCs. Considering that only 4% of stage IV CRCs are MSI-H⁸¹, a majority of *Fn*-infected CRCs with poor prognoses could be MSI-L/EMAST. These possibilities are under intensive investigation.

Conclusion

It is still questionable whether *Fn* and/or other bacterial infections cause CRC. However, cumulative evidence shows that *Fn* infection affects the course of CRC carcinogenesis linked to DNA repair and genetic/epigenetic alterations. In conclusion, *Fn*-associated CRCs are located on the right side of the colon, are inflamed by ROS, and are associated with CIMP/MSI-H and MSI-L/EMAST^{9,10,82}. *Fn*-associated CRCs exhibit shorter RFS. Mechanistically, DNA damage caused by ROS triggers MSH2/MSH6-dependent repair that results in CIMP and in MSI-H when *MLH1* is silenced, while it also induces translocation of MSH3 from the nucleus to the cytoplasm, resulting in MSI-L/EMAST. Poor prognoses for patients with *Fn*-associated CRC could be explained by *Fn*-

induced chemoresistance and/or immune evasion and/or immune suppression. It would be worthwhile to conduct further studies, especially into the detection, prevention, and treatment of *Fusobacterium*.

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Conflicts of Interest

There are no conflicts of interest.

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